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The overall goal of this research is to gather detailed information about the cellular distribution and activities of components of the uPA-system in relation to scavenger receptors and RAP in normal and breast cancer cells. We have especially noted a number of differences between the cell lines in either expression levels of these proteins or their localization. Scavenger receptors show higher expression levels in estrogen-insensitive compared to estrogen-sensitive and normal cells. Expression of both receptors seems to correspond with the malignant phenotype of these breast cancer cell lines. Immunocytochemical and immunochemical studies documented similar distribution pattern and expression levels for RAP in all cell lines. Thus, a role for RAP in affecting scavenger receptor expression in tumor development seems unlikely. Expression of uPAR is considerable higher in breast cancer cells as compared to normal cells. Occupied uPAR is found on the basal surface of the cells whereas non-occupied uPAR was mainly distributed on the apical cell surface. In estrogen-insensitive cell lines, uPA shows a very similar distribution as uPAR. Immunocytochemically, PAI-1 can be detected in the extracellular matrix of breast cancer cells but not of normal cells suggesting that expression of PAI-1 may directly correlate with the cancerous phenotype of breast cancer cells.

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## PROGRESS REPORT

## A. STATEMENT OF COLLABORATION

Work funded by this fellowship was done in the laboratory of Dr. Marilyn G. Farquhar. At the beginning of my funding period Dr. Farquhar also received funding through the U.S. Army Breast Cancer Research Program in the form of a Research grant (#DAMD-96-1-6317). Since my fellowship only pays for my salary support, the expenses of my research were covered by Dr. Farquhar's grant. Thus there is a partial overlap between my Specific Aim (SA) #1 and the SAs #1 and #2 in Dr. Farquhar's proposal in regard to the subcellular localization of scavenger receptors, urokinase-type plasminogen activator (uPA), uPA receptor (uPAR), plasminogen activator inhibitor (PAI-1), and the receptor-associated protein (RAP). Dr. Kuemmel, a postdoctoral fellow working in Dr. Farquhar's laboratory, carried out the immunocytochemical studies, and I concentrated on the immunochemical aspects of our proposals during this first year of funding. Ms. Popa, a graduate student in our laboratory was involved in the preparation of anti-human megalin antibodies. Due to my experience and knowledge of the established immunochemical and immunocytochemical techniques in Dr. Farquhar's laboratory I was substantially involved in guiding Dr. Kuemmel and Ms. Popa through these protocols and in designing experiments necessary for their work on breast cancer cells.

This decision to combine our effort increased our productivity and, as this report and Dr. Farquhar's report will clearly document, generated a significant amount of novel insight on the role of scavenger receptors in breast cancer cells and in particular about their role in the uPA-system.

## B. BODY OF WORK

### I. Preparation, purification, and characterization of polyclonal antibodies against human uPAR, human LRP, and human megalin. (In collaboration with Dr. Kuemmel and Ms. Popa)

In the beginning of our funding period we evaluated the potential of our antibodies, made against human uPAR and human LDL-receptor-related protein, LRP, to detect their respected antigens in a variety of breast cancer cells. The preparation of a new anti-human megalin antibody was time consuming and led to the present situation where we have just begun to characterize this antibody in breast cancer cells.

Polyclonal antibody (PAb) sera against the ectodomain of human LRP (1) and against the cytoplasmic domain of human LRP (1) were previously available in our laboratory as well as a polyclonal antiserum made against a recombinant human uPAR made in bacteria. First, we used aliquots of all three antisera to prepare an ammonium sulfate precipitate using a published protocol. In brief, sera were centrifuged at 3000 x g for 30 min and recovered supernatants were stirred gently at room temperature. Saturated ammonium sulfate was slowly added to bring the final concentration to 50% saturation and incubated overnight at 4°C. Precipitates were collected by centrifugation at 3000 x g for 30 min, pellets were resuspended in PBS, and solutions were dialyzed against three changes of PBS overnight.

Antibodies against the cytoplasmic sequence of LRP and anti-uPAR antibodies were further purified by affinity chromatography. For anti-LRP we used the peptide which was originally used for immunization to make an affinity column. The EDC/ Diamino-dipropylamine Immobilization Kit from Pierce was used to couple the LRP peptide via its carboxyl groups. The column was incubated with the ammonium sulfate IgG preparation and the bound antibodies were eluted according to the manufacturer's instructions.

For the affinity purification of the anti-uPAR antibodies recombinant human uPAR was coupled to Affigel-10 beads from BioRad via its lysine residues. The column was incubated with the ammonium sulfate IgG preparation and bound antibodies were eluted according to the manufacturer's instruction.

As a first step in the preparation of anti-human megalin antibodies, a homogenate of human kidney cortex was incubated in 10 mM CHAPS to extract proteins. An anti-rat-megalin PAb (1) made against a peptide sequence of the cytoplasmic domain of rat megalin was coupled to Affigel-10 beads from BioRad via its lysine residues according to the manufacturer's instructions. It was shown previously in our laboratory that this antibody was able to recognize human megalin by immunoprecipitation and immunofluorescence. The extracted proteins from human kidney cortex were applied to this affinity column to isolate human megalin. The preparation of the column, the incubation with the protein extract, and the elution of bound megalin were done according (2). Purified human megalin was used to immunize rabbits to produce PAb.

We have provided a detailed list of the antibodies used in this study and have indicated which assay they are the most useful (see TABLE I).

## II. INTRODUCTION

Proteases are of special importance in the pathogenesis of breast cancer because they play a key role in invasion and metastasis which requires the action of tumor-associated proteases to disrupt the tumor matix. Specifically, the urokinase-type plasminogen activator, uPA, its plasma membrane receptor, uPAR, and plasminogen-activator-inhibitor (PAI)-1 have been shown to have prognostic value in relationship to the progression of breast cancer where uPA plays a key role in converting inactive plasminogen to plasmin -- a highly active protease capable of degrading most extracellular proteins (3, 4). Increased activity of uPA has been linked to cell migration and invasion during embryogenic development, wound healing, and invasive growth and metastasis (3). Its proteolytic activity is blocked by PAI-1 which is also synthesized and secreted by tumor cells. Overexpression of uPA, its receptor, uPAR, and the uPA inhibitor, PAI-1, in primary tumors could be linked to an increased metastatic potential of these tumor cells.

Recently, numerous studies clearly indicated that uPA, uPAR, and PAI-1 together play an important role in the pathogenesis of breast cancer. Recent studies have reported that significantly higher levels of uPA and PAI:1 are found in mammary carcinomas than in their normal mammary epithelial cell counterparts (5-11) indicating a disturbance of the very delicate balance between activation and inactivation of uPA in tumor cells. It is known that this increase in tumor-associated proteases promotes invasion and metastasis through the dissolution of the surrounding basement membranes and tumor matrix.

The overall purpose of this work is to define the mechanisms responsible for the increased expression of PAI-1, uPA, and its receptor in breast cancers associated with increased potential for recurrence and metastasis. The working hypothesis to be tested is that the delicate balance between protease activation, inactivation and clearance is upset in breast cancers with high metastatic potential. Recently discovered scavenger receptors, i.e., LRP and megalin (12-14), have been described to bind and to endocytose uPA:PAI-1 complexes (15-20).

Both of these receptors are members of the LDL-receptor gene family, have a very similar overall structure in their extracellular domain, bind similar ligands, and bind RAP. RAP is a chaperone-like 39kDA protein with the interesting and functionally important property that it prevents ligand binding to these receptors (15, 21). Based on their ability to bind and clear uPA:PAI-1 complexes which are formed on uPAR, these scavenger receptors are thought to play a significant role in biologic and pathologic processes involving tissue remodeling, i.e., embryonic development, wound healing, and malignant cell invasion.

## III. DETAILS OF PROGRESS

Our working hypothesis was that the increased expression of uPA, uPAR, and PAI-1 in breast cancers with high metastatic potential is due to abnormalities in the clearance of these molecules by scavenger receptors.

Work has been carried out under each specific aim as follows.

### 1. SPECIFIC AIM #1 (Year 1): Determine the localization of the scavenger receptors and RAP in normal and tumor-derived mamary epithelial cell lines.

**Background information from previous work:**

Previous work has established that normal human mammary epithelial cells express RAP and either megalin or LRP (22, 23). At steady state the majority of the receptors are localized at the cell surface in clathrin-coated pits, whereas RAP is predominantly found intracellularly and in the rough ER (2, 13, 22, 24, 25).

#### a. Immunocytochemical Localization of Scavenger Receptors and RAP

**Cell Lines:** From the cell lines listed in the original proposal we selected one normal mammary epithelial cell line (184-B5, (26)) and three mammary carcinoma cell lines, MDA-MB-231 and Hs578T (estrogen insensitive) and MCF-7 (estrogen sensitive). In the order listed here these cell lines show a decreasing metastatic and invasive potential. We initially proposed to use the Hs578Bst cell line, the normal counterpart to Hs578T cells, but this was changed due to the fact that this cell line is of myoepithelial origin whereas all the cell lines listed above are derived from an infiltrating ductal carcinoma. Since the 184-B5 cell line was also established from a ductal epithelium it better fits the requirements of a control cell line.

**Methods:** The methods used were the same as those used in our recent studies of the distribution of megalin, LRP, and RAP in various cell lines (1, 25, 27). For more detailed information on specifics of method and antibodies see Dr. Farquhar's Progress Report #DAMD-96-1-6317.

Immunofluorescence: In brief, cultured cells were fixed either in paraformaldehyde or in paraformaldehyde-lysine-periodate (PLP). Incubation with primary antibodies was followed by FITC- or Texas Red-conjugated secondary antibodies. Where indicated in the figure legends cells were permeabilized with 0.5% Chaps.

Immunoelectron microscopy: In brief, cells were fixed in PLP or a mixture of paraformaldehyde/glutaraldehyde or sequentially in different concentrations of paraformaldehyde alone. Cells were cryoprotected by infiltration with sucrose and processed for cryosectioning as described (28, 29). Details of the sequential immunogold staining of the ultrathin cryosections are given in recently published papers (1, 25, 30, 31). In some instances we also have used an immunoperoxidase labeling procedure as described previously (2, 25, 32).

**Results (see TABLE II):** Immunofluorescence: All cell lines tested expressed LRP and RAP. **LRP** was found in a coated pit-like punctate distribution at the cell surface. Intracellular staining was concentrated in the Golgi region. In all cases **RAP** was found in its usual location in the ER where it colocalized with the ER marker, protein disulfide isomerase (PDI). By contrast, **megalin** was not detectable in normal but only in tumor-derived cells where it also demonstrated a punctate distribution. Its expression was the highest in Hs578T>MDA-MB-

231>MCF-7 cells. Interestingly, in Hs578T cells megalin was frequently seen at the leading edge of migrating cells.
Immunoelectron microscopy: Localization at the EM level varified that LRP is present in clathrin-coated pits and that LRP is detectable in Golgi cisternae and associated vesicles.
For more detail see Figures #1-4 in Dr. Farquhar's Progress Report #DAMD-96-1-6317.

**Conclusions:** No differences in expression or distribution of LRP and RAP were found between breast cancer cells as compared to normal epithelial cells. Curiously, megalin was expressed in the tumor cell lines but could not be detected in normal mammary epithelial cells. The level of megalin expression was greatest in estrogen-insensitive cell lines where megalin was frequently seen at the leading edge of migrating cells. We are in the process of testing more normal mammary cell lines to see if this observation is general.

**Summary of Results and Questions Answered:** These results established that all the breast cancer cell lines examined express LRP, megalin, and RAP. We showed that there are differences in the expression and distribution of megalin and RAP but not LRP between the normal mammary epithelial cell line and tumor-derived cell lines. Of particular interest is that megalin is only expressed in breast cancer cell lines and was not detectable in normal cells. No differences in expression and distribution of RAP in either normal or cancerous cells could be detected. My goal is to determine whether there are differences in the expression levels of these proteins between normal and estrogen-sensitive and/or estrogen-insensitive breast cancer cells which could explain the increased levels of components of the uPA-system in tumors. Hypothetically, a decreased expression of scavenger receptors at the cell surface could lead to a accumulation of uPA and PAI-1 at the cell surface due to decreased clearance of these complexes.

## 2. SPECIFIC AIM #2 (Year 2): Determine the levels of expression of the scavenger receptors and RAP in normal and tumor-derived mammary epithelial cells.

**Background information from previous work:**
The expression of scavenger receptors has not been studied previously in normal or tumor-derived human mammary cell lines. Therefore, the expression levels of megalin, LRP, and RAP will be evaluated in the cell lines listed above and will be compared by quantitative immunoblotting and immunoprecipitation.

### a. Total Cellular Expression:

First, we used the four mammary cell lines to compare expression levels of scavenger receptors and RAP by immunoblotting. Methods were used as described previously (2, 27).

**Methods:** In brief, proteins from confluent cell monolayers were extracted in 10 mM CHAPS and equal amounts of protein were separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) Immobilon-P membranes for Western blot analysis. PVDF membranes were incubated with primary antibodies against RAP and LRP and processed using an Enhanced Chemiluminescence (ECL) detection kit for semi-quantitative assessment of protein expression. For the immunoprecipitation of megalin with three different antibodies against rat megalin, Hs578T cells were metabolically labeled with $^{35}$S-methionine and $^{35}$S-cysteine (0.1 mCi/ml) following protocols described before (1, 27). Proteins were extracted in 10 mM CHAPS and aliquots were processed for immunoprecipitation with antibodies against rat megalin. Immunoprecipitated proteins were separated on SDS-PAGE and gels were exposed to film.

**Results (see TABLE II and Figure 1):** In this semi-quantitative approach the results show that all breast cancer cells and the normal mammary cell line express **RAP** in very similar amounts (Fig. 1B). In contrast, the expression levels of **LRP** varied significantly between these cell lines with MDA-MB-231>>>Hs578T>>184-B5>>>MCF-5 cells. The amounts expressed in MCF-7 cells were ~10X lower than that of 184-B5 cells (Fig. 1A)

In a first approach to immunoprecipitate **megalin** from metabolic labeled Hs578T cells with anti-rat megalin antibodies, all antibodies detected very similar amounts of megalin in these cells (Fig. 1C). To exclude any cross-reactivity with LRP we precipitated also with an anti-LRP antibody and found the LRP-specific band being considerable lower than the one for megalin as expected. The analysis of megalin expression in all breast cancer cell lines and in the normal cell line is in progress.

**Conclusion:** These results clearly indicate that normal and tumor-derived cells express comparable amounts of RAP and that the expression of LRP significantly varied between the cell lines. The expression of RAP does not show a correlation with the degree of malignancy of these cell lines. However, LRP expression in breast cancer cells is highest in estrogen-insensitive MDA-MB-231, which is the most metastatic and invasive cell line tested in our experiments, and LRP expression is the lowest in estrogen-sensitive MCF-7, which is the least aggressive cell line tested here. Since megalin was not found in the normal cells using immunocytochemistry but was detected in Hs578T cells in immunofluorescence and immunoprecipitation we are now testing the other mammary tumor cell lines for expression of megalin to establish a correlation between its expression and malignancy of these cells.

**b. Cell Surface Expression:**

**Methods:** Confluent cell layers of normal and tumor-derived mammary epithelial cells were surface radioiodinated following the lactoperoxidase method as described in our previous work on scavenger receptors (1). Cells were incubated at 4°C in buffer containing lactoperoxidase (10U/ml) and $Na^{125}I$ (1.0 mCi). The labeling reaction was started by the addition of 0.1% $H_2O_2$. Proteins were extracted in 10 mM CHAPS and aliquots were processed for immunoprecipitation with antibodies against LRP. Immunoprecipitated proteins were separated on SDS-PAGE and gels were exposed to film.

**Results (see TABLE II and Figure 3):** The results for **LRP** demonstrate that the number of receptors expressed at the surface of the two most malignant, estrogen-insensitive cell lines is much greater than on the estrogen-sensitive, MCF-7, cell line with MDA-MB-231>> Hs578T>>>MCF-7.

**Conclusion:** Although, immunoprecipitation results on surface expression of LRP in normal mammary cells are not available yet, the surface expression pattern in breast cancer cells seems to reflect the relative amounts of this receptor as detected in total cell lysates. This could indicate that there are no abnormalities in the traffic of LRP to the cell surface between these tumor-derived cell lines.

**3. SPECIFIC AIM #3 (Year 2): Compare the expression and cellular distribution of PAI-1, uPA, and uPAR in relation to scavenger receptors in normal mammary epithelial cells vs. tumor-derived cell lines. (Partly in collaboration with Dr. Kuemmel).**

**Background from previous work:**

It was reported recently that expression of uPA, uPAR, and PAI-1 are higher in breast cancers with metastatic potential than in normal breast tissue. Immunohistochemistry studies showed that PAI-1 (9, 33, 34) and uPA (13, 33, 34) are both detectable at the cell surface as well as in the cytoplasm of mammary tumor cells. PAI-1 was also detected in the extracellular matrix (basement membrane) (9). In normal cell lines localization of uPAR seem to be limited to focal adhesions. However, in MCF-7 cells uPAR has been localized at the leading edge of migrating cells (35), suggesting an altered distribution of uPAR in carcinoma cells.

**a. Localization of uPA, uPAR, and PAI-1**

We used the four cell lines listed under SA#1 to study the distribution of uPA, uPAR, and PAI-1 in immunofluorescence. For these studies we obtained commercially available antibodies raised against human uPA, PAI-1, and uPAR (Am. Diagnostica) and also raised a polyclonal antibody against bacterial expressed denatured recombinant human uPAR.

**Methods:** Cells were prepared for immunocytochemistry as described under Specific Aim #1. For details see legends to Figures 7 and 8 in Dr. Farquhar's Progress Report #DAMD-96-1-6317.

**Results obtained with anti-uPAR antibodies (see TABLE III):** Although, we detected expression of uPAR in all breast cancer cell lines the staining pattern varied depending on which antibody was used. Both monoclonal antibodies from American Diagnostica (# 3936 and #3937) were described to recognize unoccupied uPAR but only one (#3937) could recognize occupied uPAR (uPAR:uPA). Staining with #3936 gave a punctate pattern at the plasma membrane as did #3937 in all tested cell lines. In addition, antibody #3937 resulted in a staining pattern on the basal (facing the dish) surface of cells resembling a focal adhesion-like pattern. A gradient in the intensity of this staining pattern indicated the strongest staining in MDA-MB-231 and Hs578T and less in MCF-7>184-B5 cells. Only the PAb against uPAR detected the receptor at the surface as well as in intracellular compartments, i.e., the ER.
For more detail see Figure #7 in Dr. Farquhar's Progress Report #DAMD-96-1-6317.

**Conclusion:** In our immunocytochemical studies both normal mammary epithelial cells and tumor-derived cell lines express uPAR. The distribution pattern of occupied and unoccupied receptor seems to differ indicating a redistribution of occupied uPAR to the basal surface of these cells. To address if uPAR is localized with focal adhesions, I will apply double antibody labeling techniques together with my experience in confocal laser scanning microscopy (1, 36) and determine the degree of colocalization between occupied uPAR and marker proteins for focal adhesions. The results obtained with the PAb against uPAR were different. Since this antibody was raised against a denatured recombinant protein our results suggest that the antibody can detect precursor forms of uPAR, as present in the ER, whereas the MAb only recognize the mature surface located form of uPAR. This information can be used to design experiments to investigate the biosynthesis and exocytic trafficking of uPAR in cancerous cells compared to normal mammary epithelial cells.

**Results obtained with antibodies against uPA and PAI-1 (see TABLE III).** We are currently investigating the distribution of uPA and PAI-1 using commercially available MAbs (Am. Diagnostica) against uPA and PAI-1 in immunocytochemistry experiments. In MBA-MB-231 and Hs578T cells uPA was found in a punctate pattern along the apical membrane as well

as the basal surface in a focal adhesion-like pattern very similar to that observed with the anti-uPAR antibody which recognizes occupied uPAR. PAI-1 was found in fibrillar extracellular matrix-like material at the basal surface of these cells. Staining patterns were different in the normal mammary epithelial cell line 184-B5 in that uPA was not found in focal adhesions, only on the apical surface. PAI-1 was not detected in the normal cell line.
For more detail see Figures #8 in Dr. Farquhar's Progress Report #DAMD-96-1-6317.

**Conclusion:** These results suggest that uPA, PAI-1, and uPAR are all located in focal adhesions in malignant cells but not in normal cells. Immunoelectron microscopy is in progress to further examine this observation.

### b. Expression of uPAR Confirmed by Immunoblotting and Immunoprecipitation

**Methods:** Immunoblotting: Cell protein extracts were prepared for immunoblotting as described for the studies on scavenger receptors and RAP. For the detection of uPAR I used the PAb raised against the denatured recombinant protein since this antibody was more reactive in Western blotting than the monoclonal antibodies. Immunoprecipitation: Cells were surface radiolabeled using the lactoperoxidase method as described before. Immunoprecipitation was performed with PAb and MAb against uPAR.

**Results (see TABLE III and Figure 2):** Immunoblotting: This semi-quantitative approach showed that there are only minor variations in the expression of uPAR in these breast cancer cells. However, the tumor-derived cells express significant more uPAR than the normal mammary cell line with MDA-MB-231=MCF-7 ≥ Hs578T>>184-B5 (Fig. 2).
Immunoprecipitation: Although, the results clearly showed that all the tumor-derived cell lines express uPAR at the cell surface it was impossible to compare these amounts due to unexpected aggregation in the immunoprecipitated material. Proteins such as uPAR with a glycosylphosphotidylinositol lipid anchor tend to aggregate and precipitate when placed in detergents. Most of these aggregates did not enter the SDS-PAGE gels. Currently, I am attempting to prevent this aggregation by testing different detergents in the extraction protocol, i.e., Triton-X 100, ß-D-octylglucoside, SDS-containing buffers. I will also test the application of reagents, i.e., iodoacetamide, which prevent the reformation of disulphate bounds after boiling the precipitated proteins in reducing agents.

### c. Expression of uPA and PAI-1 Confirmed by Immunoblotting

The expression and secretion of uPA and PAI-1 by breast cancer cells and normal mammary epithelial cells is currently under investigation. Since the MAb gainst uPA and PAI-1 are originally described for immunocytochemistry we tested them first in immunofluorescence as described above. We will test these antibodies by immunoblotting and immunoprecipitation using the human fibrosarcoma cell line, HT-1080, which is a cell line that has been shown to secrete uPA and PAI-1.

**Summary of Results and Questions Answered:** We demonstrated that the level of expression of uPAR, uPA, and PAI-1 are increased in the breast cancer cells tested as compared to the normal mammary cell line. The proteins are expressed either on the cell surface (uPAR and uPA) or in the extracellular matrix (PAI-1) and their distribution differs between tumor-derived and normal mammary cells. We have shown that unoccupied uPAR is distributed over the entire apical cell surface but that the localization of occupied uPA-bound uPAR seems to be restricted to focal adhesions. Next, we will move on to precisely localize the components of the uPA-system at the cell surface at the EM level.

**4. SPECIFIC AIM #4 (Year 3): Determine the fate of uPA, uPAR, scavenger receptors, and uPA:PAI-1 complexes at the cell surface of normal and tumor-derived mammary epithelial cells**

**Background from previous work:**

Recently, a model has been proposed for the metabolism of uPA:PAI-1 complexes by scavenger receptors (21). Therein, uPA is secreted and bound by the uPAR on the leading ege of the migrating cells. uPA activity is regulated and inactivated by complexing with PAI-1. This uPAR:uPA:PAI-1 complex is then cleared from the cell surface via scavenger receptors (37-40). However, in breast cancers with metastatic potential there is increased expression of uPAR, uPA, and PAI-1 suggesting an abnormality in the binding and/or clearance of the complexes via scavenger receptors. The studies carried out under Specific Aims #2 and 3 have established that there are abnormalities in the distribution and expression of uPAR, and the studies in this specific aim are designed to establish whether there are abnormalities in their clearance function, i.e., internalization and endocytic trafficking.

First we must compare LRP and megalin expression levels between tumor-derived and normal mammary epithelial cells. Furthermore, we must determine the number of functional binding sites for uPA:PAI-1 complexes on these cells to compare the receptor-specific ligand binding activity in tumor-derived cells versus normal cells.

**a. Quantitation of uPAR and Scavenger Receptors at the Cell Surface**

Our results so far documented that the breast cancer cell lines express uPAR and scavenger receptors. To establish a correlation between the malignancy of these cancerous cells and the expression of these receptors it is neccessary to 1) quantitate the amount of receptor present at the cell surface, and 2) to determine if the receptor functions normal in all cell lines.

**1) Assessment of Functionality of Scavenger Receptors**

The goal was to determine if scavenger receptors show binding affinities and receptor-mediated endocytosis of their specific ligands as known from studies on non-cancerous cells. α2macroglobulin (α2M) represents a unique ligand for LRP that is not taken up by megalin or any other receptor. In contrast, all ligands used for binding and endocytosis studies on megalin so far can also bind to other members of the LDL-receptor family including LRP. Therefore, the only results obtained so far are with LRP.

**Methods:** Quantitation of binding capacities: α2M was radiolabeled as described previously (27) and bound to normal and tumor-derived cells at 4°C to prevent internalization. After washing, bound radiolabeled protein was determined by direct gamma counting (counts/min (cpm)). These readings were divided by the specific activity of the radiolabeled ligand (cpm/ng protein) to quantitate the molar amount of ligand bound to the cell surface. These amounts were normalized to total cellular protein to allow a direct comparison between normal and cancerous cell lines. Quantitation of binding affinities: Cells were incubated at 4°C with radiolabeled ligand in saturating concentrations. The binding was competed by co-incubation with increasing amounts of unlabeled ligand. By definition, the concentration of unlabeled ligand that results in 50% binding inhibition represents the binding affinity (Kd). Endocytic function of LRP: Radiolabeled ligand was bound to cells at 4°C and receptor/ligand complexes were internalized following incubation at 37°C. At various times the incubation media were collected and protein was precipitated in 10% trichloroacetic acid. Radioactivity in the non-precipitable material was measured by gamma counting. The results reflect a direct measurement of lysosomal degradation of $^{125}$I-α2M over time. This protocol is a well established protocol for quantitating receptor-mediated endocytosis.

**Results (see Figures 4, 5, 6):** In our experiments we show a great variation between the amounts of functional LRP expressed at the cell surface of normal and tumor-derived cells. Although, MDA-MB-231 cells have 6-17x more functional receptors on their surface than Hs578T, MCF-7, and 184-B5 cells (Fig. 4) the three breast cancer cell lines show an identical affinity for α2M binding to LRP (Kd=0.45 nM). That value is slightly lower than the one determined for the normal cell line (Kd=0.14 nM) (Fig. 5). When degradation of $^{125}$I-α2M was measured, Hs578T and the normal mammary epithelial cells showed degradation in a linear, time-dependent manner indicating that LRP is constitutively recycled for successive rounds of ligand uptake (Fig. 6). The estrogen-sensitive cell line MCF-7 showed a notable low degradation rate consistant with their low level of LRP expression. Interestingly, MDA-MB-231 cells showed rapid degradation of $^{125}$I-α2M followed by very little degradation over the remaining 6 hour time course.

**Conclusion:** The amount of function LRP expressed on the cell surface of each cell type as determined by quantitating $^{125}$I-α2M binding closely correlates with the quantitative data we obtained for LRP expression by immunoblotting and immunoprecipitation analyses. LRP expression in Hs578T and normal mammary epithelial cells are comparable, and very little LRP is found in MCF-7 cells. MDA-MB-231 cells express a significant amount of functional LRP on the surface which would explain the large amount of α2M endocytosed and degraded in the very beginning of the incubation period. However, the rate of ligand degradation following this initial internalization step is low. These results suggest that in MDA-MB-231 cells this receptor undergoes one rapid round of internalization to deliver its bound ligand to lysosomes, and then is incapable of recycling to the cell surface for additional rounds of ligand uptake. This could reflect an abnormality in the mechanism of LRP recycling in this cell line. Although, the binding affinities for LRP in normal and cancerous cells is slightly different it is safe to conclude that no severe deficiencies are present in the binding properties of the receptor in the tumor-derived cells. Next it will be neccessary to establish the exact time course for the recycling of LRP in all four cell lines to clarify if our results on MDA-MB-231 cells truely reflect a abnormality in the recycling of this receptor.

**2) Assessment of Functionality of uPAR**

Due to the unique mechanism of interaction between uPA and uPAR on one hand and between uPA:PAI-1 complexes and LRP on the other, the ligand binding and ligand uptake experiments as used for studies on LRP have to be adjusted for the studies on uPAR.

Specific ligand for uPAR: The most specific ligand for human uPAR is human uPA based on the high species specificity between this protease and its receptor. The binding affinity of uPA to LRP is insignificant low. However, in the presence of PAI-1, which is secreted by our tumor-derived cell lines, uPA:PAI-1 complexes rapidly form on uPAR. This process would immediately release uPA in the form of uPA:PAI-1 complexes from the receptor and is therefore not a suitable ligand for our binding studies on uPAR. I am in the process of preparing an aminoterminal fragment (ATF) of uPA as a recombinant fusion protein. ATF consists only of the binding site for uPAR and will neither show the proteolytic activity nor possess the binding site for PAI-1 as the full-length uPA. In brief, total RNA was isolated from a human cell line (U937) using reverse-transcriptase-PCR to generate and amplify the specific cDNA for human uPA. I cloned the ATF by PCR using this cDNA as template. In a second step, I constructed two plasmid vectors containing a c-myc sequence which is attached to the C-terminal end of ATF. Vectors will be suitable for prokaryotic as well as eukaryotic expression. Radiolabeled ATF will be bound to the cell surface exclusively to uPAR and will allow us to determine binding capacities and binding affinities using protocols as decribed for the same experiments performed on LRP.

## IV. OVERALL CONCLUSIONS

In the first year of our proposal we have made significant progress in characterizing the expression levels and subcellular distribution of the scavenger receptors (LRP and megalin), RAP, uPAR, uPA, and PAI-1 in three breast cancer cell lines and one normal mammary epithelial cell line. We have especially noted a number of differences between the four cell lines in either expression levels of these proteins or their localization. The following conclusions highlight our results over the past year.

1. Quantitating LRP expression levels in the highly metastatic estrogen-insensitive breast cancer cell line, MDA-MB-231, by immunoblotting, immunoprecipitation, and ligand binding demonstrates that these cells express 10X higher levels of LRP than the estrogen-sensitive breast cancer cell line, MCF-7, or the normal mammary cell line, 184-B5.

2. Megalin is expressed in breast cancer cell lines but could not be detected in the normal cells. We need to investigate this further to determine if megalin expression is a result of the cancerous phenotype or plays a causal role in tumor development.

3. The expression levels and ER localization of RAP are similar in all cell lines tested. From our data it is safe to conclude that RAP is unlikely to play a role in tumor development.

4. Levels of uPAR are considerable higher in the three breast cancer cell lines as compared to the normal cell line. In MDA-MB-231 and Hs578T cells, we found uPAR distributed in a punctate pattern on the apical surface and possibly associated with focal adhesion sites on the basal surface. This latter observation is consistent with uPAR interacting with proteins involved in cell-matrix interactions. To address this possibility, our future plans include to better define the subcellular localization of uPAR at the EM level.

5. uPA appears to be distributed in a punctate pattern on the apical cell surface in all four cell lines. However, in estrogen-insensitive MDA-MB-231 and Hs578T cells, but not in normal cells, uPA is additionally found at focal adhesion sites in a very similar distribution pattern as seen for uPAR. These results suggest that increased proteolytic activity of uPA may be concentrated via uPAR binding at sites of cell adhesion which would greatly enhance cell detachment and migration of breast cancer cells as compared to normal cells. We will attempt a closer examination of the distribution of uPA in breast cancer cells versus normal mammary cells in the next year to further explore this possibility.

6. PAI-1 is a secreted protein that is known to associate with proteins of the extracellular matrix (ECM). Our immunocytochemical results so far show that PAI-1 is distributed extracellular in a pattern consistent with ECM deposition in all three breast cancer cell lines but is absent from the matrix of normal mammary cells. These results suggest that the expression of PAI-1 may directly correlate with the cancerous phenotype of breast cancer cells. We will perform quantitative studies to determine if any PAI-1 is being made and secreted by normal mammary epithelial cells and further evaluate PAI-1 expression levels in the breast cancer cells.

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# APPENDICES

# TABLE I

## Available Antibodies

| Antibodies | Type | Designation | Assays | | |
|---|---|---|---|---|---|
| | | | IF | IB | IP |
| anti-human LRP (recognizes the cytoplasmic tail) | PAb | anti-$LRP_{ct}$ (human) | + | + | + |
| anti-human LRP | PAb | anti-LRP (human) | + | + | + |
| anti-rat megalin (recognizes the cytoplasmic tail) | PAb | anti-$megalin_{ct}$ (human) | + | + | + |
| anti-rat megalin | PAb | anti-megalin (rat) | + | +/- | + |
| anti-rat megalin (against ligand-binding domains I or II) | PAb | anti-$megalin_{LBD\ I,II}$ (rat) | + | + | + |
| anti-human megalin | PAb | anti-megalin (human) | + | + | + |
| anti-human uPAR | PAb | anti-uPAR (human) | + | + | + |
| anti-human uPAR (American Diagnostica) | MAb | 3936 | + | nd | nd |
| anti-human uPAR (American Diagnostica) | MAb | 3937 | + | nd | nd |
| anti-human uPA (B chain) (American Diagnostica) | MAb | 394 | + | + | + |
| anti-rat RAP | PAb | anti-RAP (rat) | - | + | + |
| anti-human RAP | PAb | anti-RAP (human) | + | + | + |

+/- = of limited use
+ = useful in the indicated assay
nd = not done
PAb = polyclonal antibody
MAb = monoclonal antibody

IF = immunofluorescence
IB = immunoblotting
IP = immunoprecipitation

## TABLE II

### Expression of LRP, megalin, and RAP in normal and malignant breast cancer cells

| | LRP | | | Megalin | | RAP | |
|---|---|---|---|---|---|---|---|
| **Cell line** | **IF (perm)** | **IF (surface)** | **IB** | **IF** | **IP** | **IF** | **IB** |
| 184-B5 | +++ | + | + | - | nd | +++ | + |
| MCF-7 | +++ | nd | +/- | + | nd | +++ | +++ |
| Hs578T | +++ | ++ | + | +++ | ++ | +++ | +++ |
| MDA-MB-231 | +++ | ++ | +++ | ++ | nd | +++ | +++ |

perm. = permeabilized
surface = surface labeling
IF = immunofluorescence
IB = immunoblot
IP = immunoprecipitation

\- = not detected
+/- = very weak
\+ = weak
++ = moderate
+++ = strong

## TABLE III

### Expression of uPAR, uPA, and PAI-1 in normal and breast cancer cells

| | uPAR | | | uPA | PAI-1 |
|---|---|---|---|---|---|
| **Cell line** | **IF (perm)** | **IF (surface)** | **IB** | **IF** | **IF** |
| 184-B5 | +++ | + | + | + | - |
| MCF-7 | +++ | + | +++ | - | - |
| Hs578T | +++ | +++ | +++ | +++ | ++ |
| MDA-MB-231 | +++ | +++ | +++ | +++ | +++ |

perm. = permeabilized
surface = surface labeling
IF = immunofluorescence
IB = immunoblot

\- = not detected
\+ = weak
++ = moderate
+++ = strong

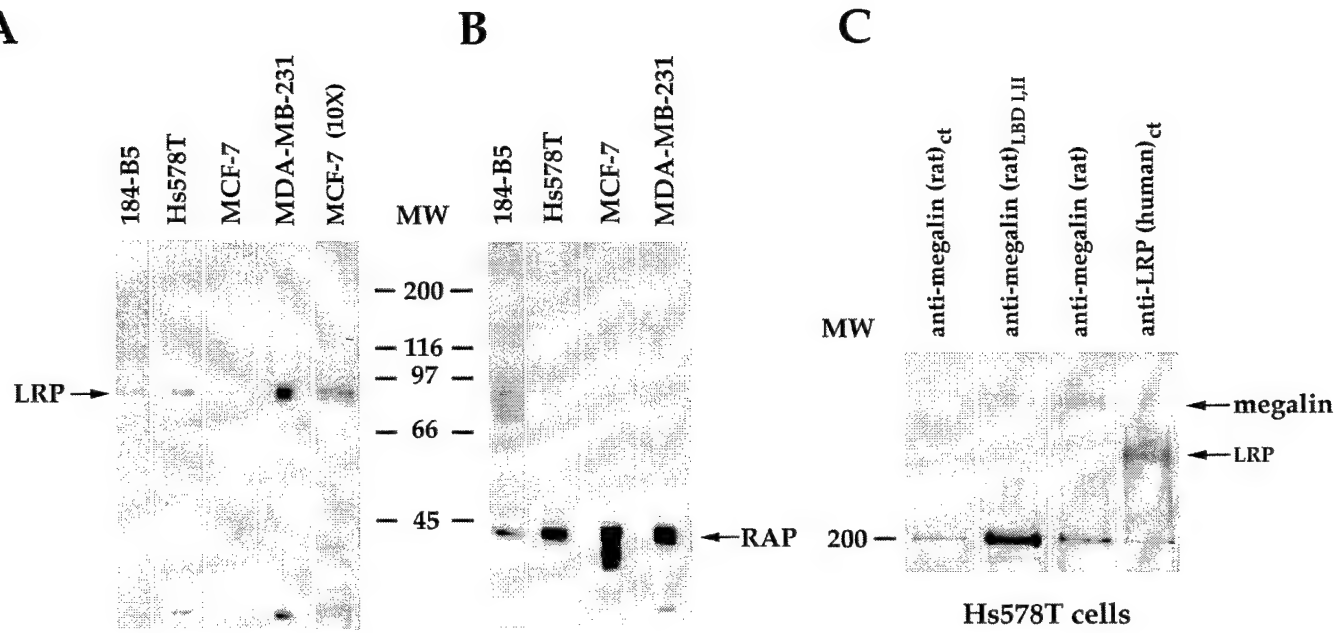


**Figure 1: Expression of scavenger receptors and RAP in normal and tumor-derived mammary epithelial cell lines.** For immunoblotting (A, B) proteins were extracted in 10 mM CHAPS, equal amounts of protein were separated by SDS-PAGE, and transferred to PVDF membranes for immunoblotting with anti-LRPct (human) and anti-RAP (human) antibodies.

**LRP** (A): Highest amounts of LRP were expressed in estrogen-insensitive tumor-derived cell lines MDB-MB-231 and Hs578T, whereas in the estrogen-sensitive cell line, MCF-7, LRP expression could only be demonstrated when the amount of protein electrophoresed was increased 10-fold (MCF-7 (10X)).

**RAP** (B): All tumor-derived cell lines expressed similar amounts of RAP and expression levels were slightly higher than in the normal cell line, 184-B5.

**Megalin** (C): Hs578T cells were metabolically labeled with 35S-methionine/cysteine and proteins were extracted in 10 mM CHAPS. Aliquots were incubated with anti-megalin (rat) antibodies bound to protein A-agarose beads. Antibody-bound proteins were processed for and separated by SDS-PAGE. Megalin was detected in the estrogen-insensitive cell line, Hs578T, with three anti-megalin (rat) antibodies.

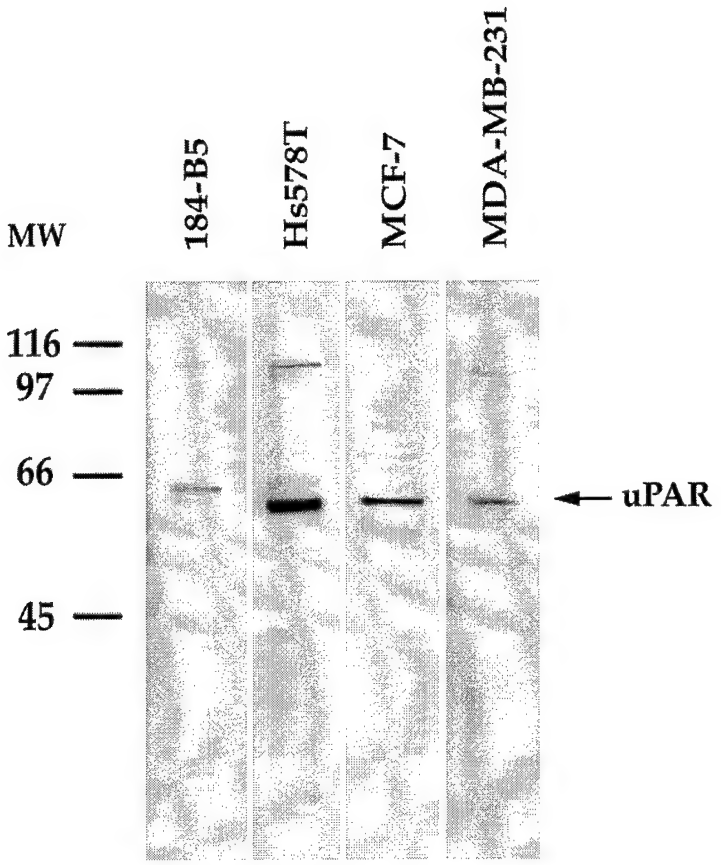


**Figure 2: By immunoblotting uPAR is expressed in normal and tumor-derived mammary epithelial cell lines.** Protein extracts were prepared and processed for immunoblotting (as in Figure 6) with anti-uPAR (human) antibodies. All tumor-derived cell lines express uPAR with no significant differences in the expression levels, but all breast cancer cell lines express more uPAR than does the normal mammary cell line, 184-B5.

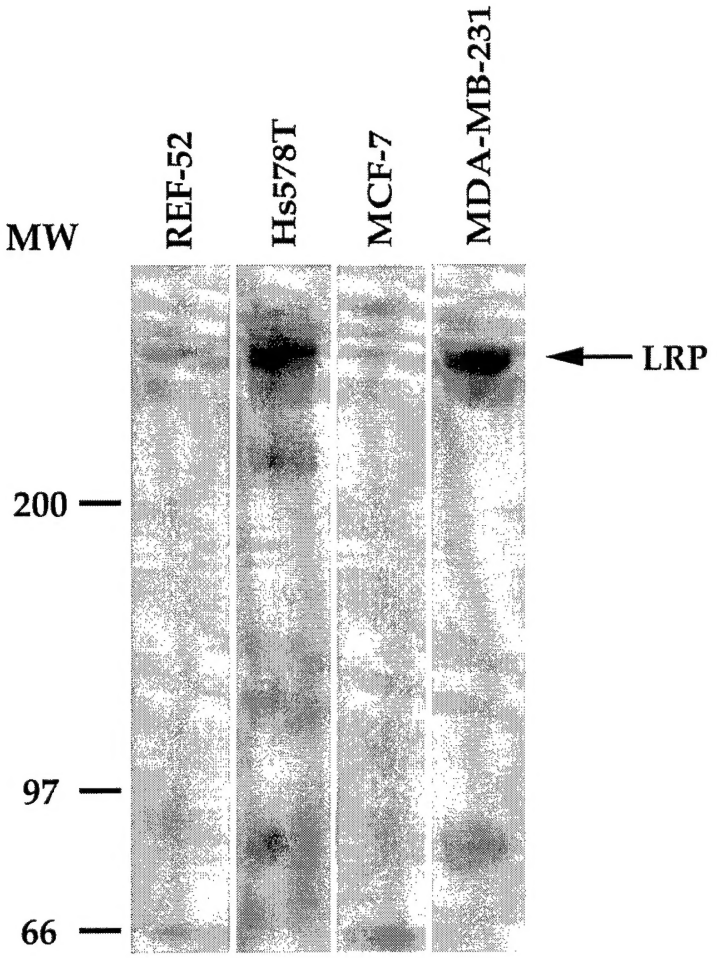


**Figure 3: Cell surface expression of LRP in tumor-derived mammary epithelial cell lines.** Cells were radiolabeled by lactoperoxidase-mediated cell surface iodination. Cells were lysed in 10 mM CHAPS and radiolabeled proteins from each cell line were immunoprecipitated using anti-LRP (human) antibodies bound to protein A-agarose beads. Precipitated proteins were processed and separated by SDS-PAGE. Expression of LRP is much greater in estrogen-insensitive MDA-MB-231 and Hs578T cells than in estrogen-sensitive MCF-7 cells where the receptor was barely detectable. REF-52 (rat embryonal fibroblasts), used as a positive control in this experiment, also expressed much lower levels of LRP than the two estrogen-insensitive breast cancer cell lines.

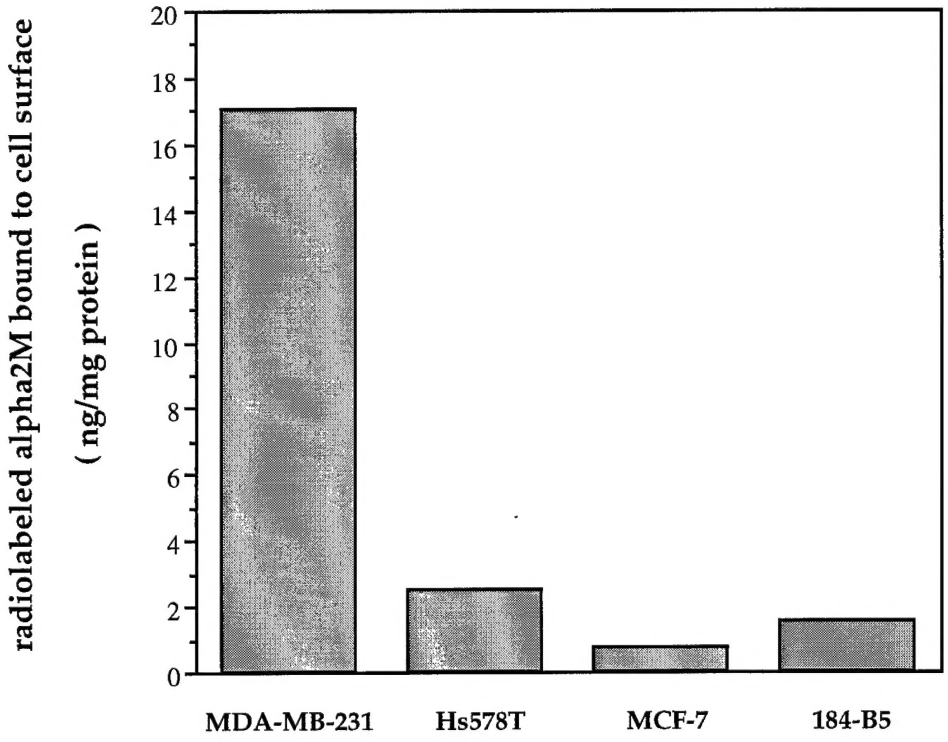


**Figure 4: α2macroglobulin (α2M) binding capacities on normal and tumor-derived mammary epithelial cell lines.** 125I-α2M was bound to cells at 4°C for 3 hr. Cell associated radioactivity was quantitated by gamma counting and normalized to total cellular protein. Binding capacity of LRP in MDA-MB-231 cells was 6-17X greater than in the normal mammary cell line or breast cancer cell lines.

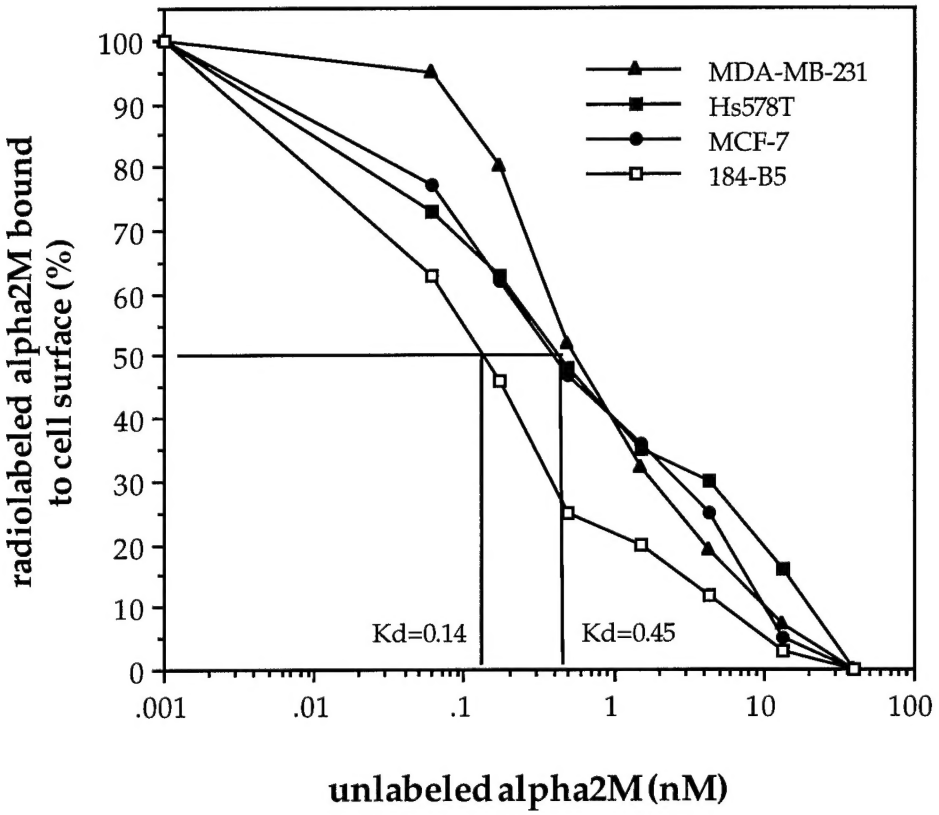


**Figure 5: α2macroglobulin binding affinities to LRP in normal and tumor-derived mammary epithelial cell lines.** Cells were incubated at 4°C with radiolabeled 125I-α2M (2 nM) in the presence of increasing amounts of unlabeled α2M (0-40 nM). The concentration of unlabeled α2M which resulted in 50% inhibition of 125I-α2M binding represents the binding affinity (Kd). All three breast cancer cell lines demonstrated a slightly lower affinity for α2M binding to LRP (Kd=0.45 nM) than the normal cells (Kd=0.14 nM).

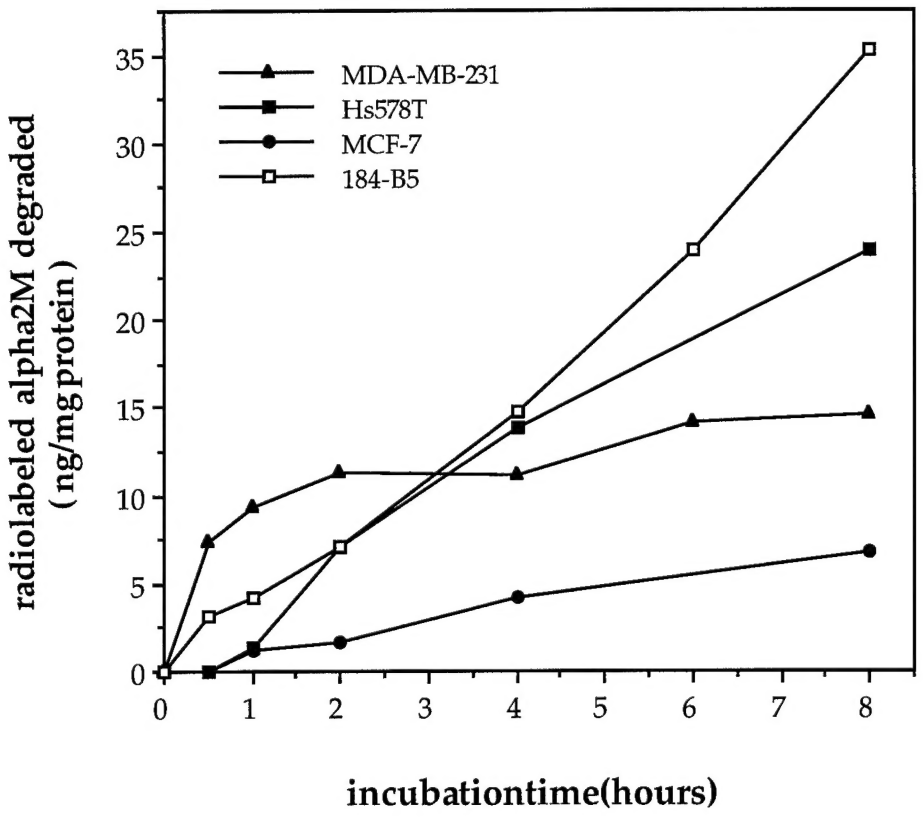


**Figure 6: LRP-mediated internalization and degradation of 125I-α2M in normal and tumor-derived mammary epithelial cell lines.** Cells were incubated with radiolabeled α2M (2 nM) at 37°C and the media sampled at various times, adjusted to 10% TCA, and non-precipitable material was measured by gamma counting. Hs578T and normal epithelial cells (184-B5) degraded 125I-α2M in a linear, time-dependent manner. MCF-7 cells showed little degradation of the ligand. Interestingly, MDA-MB-231 cells demonstrated rapid uptake and degradation of 125I-α2M followed by very little degradation over the remaining 6 hr time course.